PREVALENCE OF ANTIBODIES TO AVIAN INFLUENZA A VIRUS IN HUMANS SERA IN KHARTOUM STATE

By

Mohammed Osman Abdel Fatah Abdelrahman

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Department of Microbiology
Faculty of Veterinary Medicine
University of Khartoum

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DEDICATION

To the soul of my father

Who inspired us in our whole life.

To precious Mother.

Brothers and incredible Sister.

I dedicate this work

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First, all thanks and praises to Allah, the lord of the mankind and all past and existing creatures, and the prayers and peace are upon the prophet Mohammed the mercy message to earth.

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RNA	Ribonucleic Acid
HA	Hemagglutinin
NA	Neuraminidase
HI	Hemagglutination Inhibition
IDT	Immunodiffusion test
ELISA	Enzyme Linked Immunosorbbent Assay
VN	Virus Neutralization
WHO	World Health Organization
RBCs	Red Blood Cells
AGID	Agar Immuno-diffusion
AI	Avian Influenza
LPAIV	Low Pathogenic Avian Influenza Virus
HPAIV	Highly Pathogenic Avian Influenza Virus
OIE	Office International of Epizootics
NB	Integral membrane protein
M P	Membrane protein
RNP	Ribonucleoprotein
ATP	Adenosine Tri-phosphate
mRNA	Messenger Ribonucleic acid
vRNA	Viral Ribonucleic acid
SAEC	Sudan Atomic Energy Commission
NP	Nucleocapsid protein
NS	Non Structural Proteins
cRNA	Complementary Ribonucleic Acid
ISAV	Infectious Salmon Anemia Virus
HEFP	Hemagglutinin-Esterase Fusion Protein

MDCK	Madin-Darby canine Kidney
PMKC	Primary Monkey Kidney Cells
CFT	Complement Fixation Test
RDE	Receptor Destroying Enzyme
NI	Neuraminidase Inhibition
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
cDNA	Complementary Deoxy Ribonucleic Acid
CAM	Chorioallantoic Membrane
PBS	Phosphate Buffer Saline
DDW	De-Ionized Distilled Water
SDS	Sodium Dodecyl Sulphate
NAI	Notifiable Avian Influenza viruses
HAU	Hemagglutination Unit

ABSTRACT

A serological survey was carried out to estimate the magnitude and the prevalence of avian influenza (AI) antibodies in Khartoum state population.

A total of 222 serum samples were collected from individuals, without any apparent clinical signs of influenza or influenza like diseases, while attending the Sudanese Atomic Energy Commission laboratories in Khartoum and Omdurman. The collected sera were containing 179 female samples and 43 male samples. The collected sera were first tested by AGID test, the results showed that 76 samples out of 222 (34.2%) were positive for influenza A type.

Screening of the positive sera by hemagglutination inhibition (HI) test for sub-typing antibodies, using H5N1 and H7N3 as antigens and pretreatment of sera to remove nonspecific inhibitors, revealed that 13 out of 76 were positive for H5 antibodies and only 5 samples were positive against H7.From the positive samples to H5 there were 8 samples from Khartoum and 5 from Omdurman area, and the 5 positive sera to H7 contain 3 samples from Khartoum and the rest 2 were from Omdurman.
But due to the inability of HI test to detect low titers of antibodies to avian influenza viruses (AIV) in human sera, these results may be under-estimated.

The finding of this survey is that, there are circulating H5 and H7 viruses among Khartoum state environment, but without further investigation we can not prove are they H5N1 and H7N3 or not.

ملخص الأطروحة

فى هذه الدراسة تم اجراء مسح مصلى لتحديد مقدار وإنتشار الأجسام المضادة لبعض فيروسات انفلونزا الطيور في جزء من سكان ولاية الخرطوم باستخدام اختبارى الانتشار المناعى فى الجل لتحديد النمط المصلى و تثبيط التلازن لتحديد نوع السلالة .

تم جمع 222 عينة مصل مِن أشخاص، بدون أي إشارات سريرية ظاهرة للإنفلونزا أو أمراض لها أعراض مثل الإنفلونزا أثناء زيار مُ لمختبرات لجنةِ الطاقةِ الذريةِ السودانيةِ في الخرطوم وأم درمان . العينات التى جمعت كانت تحتوى على 179 عينةَ اناثَ و43 عينةَ ذكور .فى البدء تم فحص العينات بإختبارالأنتشار المناعى فى هلام الجل ،أوضحت النتائج بأنّ 76 عينةَ من جملة العينات (34.2 %) كانت إيجابية للإنفلونزا من النمط A .

عند فحص العينات الموجبة للنمط A بإختبار تثبيط التلازن لتحديد السلالة بإستعمال H 5 N 1 و H 7 N 3 كأنتجينات و معالجة السيرم قبل الأختبار ،لإزالة مثبطات التلازن غير النوعية، وجد بأنّ 13 عينة من مجموع 76 كانت إيجابية للأجسام المضادةِ ل H 5 و 5 عيناتٍ فقط كانت إيجابية ضد H 7 . مِن العيناتِ الإيجابيةِ ل H 5 كان هناك 8 عيناتٍ مِن الخرطوم و5 مِن منطقةِ أمدرمان، ومن الـعينات الخمس الموجبة لل H 7 كانت هنالك 3 عيناتٍ مِن الخرطوم والبقية كانت مِن أم درمان.

لكن بسبب عدمِ قابلية اختبارتثبيط التلازنً لإكتشاف معايير منخفضة مِن الأجسام المضادةِ لفيروساتِ إنفلونزا الطيور (AIV) في مصل الإنسان ، هذه النتائج قَد تكُون أعلى مما تم الحصول عليه.

خلصت هذه الدراسة الى أنّ هنالك وجود للفيروسات من النوعين H 5 **و** H 7 **فى بيئة ولاية الخرطوم، لكن بدون اختبارات آخرى فانه ليس بالإمكان اثبات ما اذا كانت هذه الفيروسات هى** H 5 N 1 **و** H 7 N 3 **أم لا.**

INTRODUCTION

Influenza A virus genus is a member of the family Orthomyxoviridae (Lamb, 1989) which include also the genera: Influenza B, Influenza C and Thogoto viruses. The genome of influenza A virus is composed of 8 RNA segments, two of these molecules are most important in classification of influenza viruses, and they are; HA segment which incodes the haemagglutinin protein, and the NA segment which incodes the neuraminidase protein. (Murphy, Gibbs, Horzinek, and Studdert. 1999).

Influenza viruses spread from person to person via small particle aerosols and then invade the cells of the upper respiratory system, bind to carbohydrates on the glycoprotein of the host epithelial cells and enter by receptor mediated endocytosis (Lamb, 1989). Since the survey done by Salim (1970) which proved the presence of (H3N2) A2 1/68/Hong Kong subtype in Sudan, there were no further researches in this subject. Also due to the last avian influenza outbreak, and the acute cases symptoms, which appear to be an influenza disease, there was a need to determine the level of influenza viruses' antibodies in Sudanese population sera.

In the reports of many authors, there is considerable variation in data for comparative studies upon diagnostic tests for detection of respective antibodies in spontaneous (Edair, Todd, McKillop And McNulty. 1989; Edair, Burns, McNulty and Todd. 1990; Arenas, Carranza, Perea, Miranda, Maldonado and Hermoso. 1990; Boer, Back and Osterhaus. 1990; Lamichhane and Kirkegaard. 1997; Zhou, Chan, Heckert, Riva and Cantin. 1998; Sala, Cordioli, Moreno-Martin, Tolles, Brocchi, Piccirillo and Lavazza. 2003; Meilin, Wang, Zhang, Zhao, Li, Tan and Chen. 2004), and experimental (Maulemans, Carlier, Gonze and Petit. 1987;

Beck and Swayne. 1997) infections. In some cases, higher positive results have been obtained in sera tested by means of hemagglutination inhibition (HI) test compared to either immunodiffusion test (IDT) or Enzyme linked immunosorbbent assay (ELISA) (Zhou et al., 1998; Sala et al., 2003).

Serological diagnosis is important when clinical specimens are not available or when the laboratory does not have the resources of virus isolation. Serological methods such as hemagglutination inhibition (HI), virus neutralization (VN) and ELISA are useful in epidemiological, immunological studies as well as in the evaluation of vaccine immunogenicity (WHO Manual, 2002).

The hemagglutination inhibition (HI) test, which detects subtype-specific antibodies against hemagglutinin (HA), is commonly used for serologic diagnosis of influenza virus infection and for determination of susceptibility to influenza virus in epidemiologic and vaccine studies.

Naturally occurring inhibitors of hemagglutination by influenza viruses present in the sera of humans and animals were first described by Francis (1947). These substances act like antibody in an HAI test by interacting with the influenza HA and thus preventing agglutination of erythrocytes (RBCs) by the virus. Three known classes of inhibitors, designated alpha, beta, and gamma, have been detected in human sera (Krizanova and Rathova. 1969).

Hemagglutination inhibition test was used to detect antibodies to H7 infection in human sera that collected from persons exposed to poultry and showed subclinical infection which revealed positive of 49% of 508 and in 64% of 63 persons exposed to A (H7) infected persons (Meijer, A., Bosman, A., van de Kamp, E.H.M., Wilbrink, B., Holle, M. D. B. and Koopmans, M. 2006). Also HI test detected H5-specific antibody in single serum samples from two of three patients with culture-confirmed

H5N1 virus infections **(**Rowe, Abernathy, Jean Hu-Primmer, Thompson, Lu, Lim, Fukuda, Cox and Katz. 1999).

Objectives:

-General Objectives:

Serosurvillance for avian influenza A virus among human in Khartoum state population.

-Specific Objectives:

1-The prevalence of the antibodies to type A influenza using agar immunodiffusion test.

2-Subtyping and titration of AGID positive sera using antigens of H5N1 and H7N3 by Hemagglutination Inhibition test.

Chapter One

LITRATURE REVIEW

1.1 Definition of influenza disease:

Influenza is an acute respiratory disease caused by the influenza virus and can spread rapidly and widely in the winter season. About 20% of children and 5% of adults world-wide develop symptomatic influenza each year (Yohannes, Roche, Spencer and Hampson. 2003). It causes a broad range of illness, from asymptomatic infection through various respiratory syndromes, to fulminate primary viral and secondary bacterial pneumonia. Epidemics and pandemics of influenza have been documented for centuries, with the first clear record dated back in 1580 (Potter. 2001).

Avian influenza (AI) is a highly contagious disease caused by type A influenza viruses that are members of the family Orthomyxoviridae in the genus Influenza A virus (OIE, 2005). Infection with low-pathogenic avian influenza viruses (LPAIV) usually is asymptomatic or it induces mild respiratory symptoms, but it can rapidly reduce egg production in poultry. Infection with highly pathogenic avian influenza viruses (HPAIV) is characterized by the sudden onset of fatal systemic disease in susceptible species. Importantly, some strains of LPAIV can serve as progenitors that rapidly mutate into HPAIV, which may infect humans, causing death, as evidenced by the recent outbreaks in Asia (Fisher, 2006).

Influenza viruses are important pathogens of animals, but only rarely are zoonotic. Instead, they are the ultimate species jumpers, for ever evolving host range variants in animals that become epidemic and even pandemic when they invade humans. Therefore, veterinary and human concerns over influenza are tightly linked. (Murphy et al., 1999).

1.2 History of the disease:

1.2.1 History of the disease in the world:

Influenza has probably existed from antiquity, although the lack of specific pathognomonic signs makes this assertion less definite than in the case of smallpox or cholera (Kilbourne, 1987). Even so, historical records of rapidly spreading of catarrhal fevers, in Great Britain, suggest that major influenza epidemics affected human populations as early as the sixteenth century. In spite the lacking of direct evidence for horse-to-human transmission of influenza viruses, the sizeable concentrations of these animals near previous centers of human population makes them attractive candidates as disease intermediaries (Hirsch, 1883). In modern times, pigs have been accorded a prominent role in the generation of major influenza outbreaks (Goldfield, Bartley, Pizzuti, Black, Altman, and Halperin. 1977; Scholtissek, Burger, Kistner, and Shortridge. 1985**)**. Until recently, direct avian-to-human transmission associated with fatal outcome in infected individuals was believed not to occur. But this was changed in 1997 when 18 individuals – six of whom died – were infected with H5N1 avian viruses in Hong Kong (Claas, Osterhaus, van Beek, De Jong, Rimmelzwaan, Senne., Krauss, Shortridge, and Webster, 1998; Subbarao, Klimov, Katz, Regnery, Lim, Hall, Perdue, Swayne, Bender, Huang, Hemphill, Rowe, Shaw, Xu, Fukuda and Cox. 1998). Since then, direct avian-to-human transmission has been reported in several other incidents, such as human infection with H7N7 avian virus in the Netherlands in 2003 (Fouchier, Schneeberger, Rozendaal, Broekman, Kemink, Munster, Kuiken, Rimmelzwaan, Schutten, Van Doornum , Koch, Bosman , Koopmans and Osterhaus . 2004 ; Koopmans, Wilbrink, Conyn , Natrop, van der Nat, Vennema, Meijer, van Steenbergen , Fouchier, Osterhaus and Bosman . 2004), or H5N1 avian viruses in Hong Kong in 2003 (Peiris, Yu , Leung, Cheung , Ng,

Nicholls, Ng, Chan , Lai, Lim , Yuen, and Guan . 2004), and in Vietnam and Thailand where 42 cases and 30 deaths were reported through September of 2004 (Tran, Nguyen, Nguyen, Luong, Pham, Mai, Nguyen , Pham , Vo Cong, Quynh , Dao, Nguyen , Hoang , Cao , Nguyen, Nguyen , San., Christiane, Tran, de Jong , Constance , Peter, Wilina, Peter, and Jeremy . 2004).

1.2.2 History of the disease in Sudan:

Influenza-like diseases have been known to occur in the Sudan for a long time, but the true nature of these diseases has not been investigated. In 1957 there was an epidemic of an influenza-like disease which physicians attributed it to the Asian influenza type of virus that causing epidemics in other countries at the same time. However, no virus isolation or serological studies were performed in the Sudan at that time. In the winters of 1968-69 and 1969-70, there were epidemics of an influenza-like disease throughout the country, and laboratory investigations were carried out to establish the nature of the causative organism(s) of these epidemics (Salim, 1971). The first description of avian influenza disease was reported in 1925 on Sudan Veterinary Services annual report. But all of experimental surveys among fowl with respiratory diseases, to isolate the virus, were unable to isolate the virus (El Mubarak, 1970).

Then, Gafar and Kheir (1985) reported influenza ribonucleic protein antibody in the sera from different animal's species; camel, goat, sheep and cattle at eastern Sudan- Kassala using agar-gel immuno-diffusion test (AGID). Avian influenza (AI) antibodies have been detected in Sudan by (Manal, 2000 and Wegdan, Kheir and Ballal. 2007). Manal used AGID and Competitive ELISA tests to investigate the presence of avian, H1N1 and H3N2, subtypes in some of Khartoum state chickens farms, and she

found that 28.4% of test sera were positive for influenza type A. On the other hand, Wegdan used indirect ELISA to detect influenza type A antibodies in chicken's sera collected from different regions of Sudan. Wegdan detect A1 virus type A antibodies in 911 serum samples out of 1054(86.4%) in young and adult chickens. On the eighth of May 2006, the OIE reported the presence of H5N1 avian influenza in Sudan. Outbreaks have been reported in Atbara in River Nile State and in four poultry farms in Gezira State. Further information about the outbreaks has been requested by the OIE, and this was the first confirmation of H5N1 infection in Sudan, although the presence of the H5 subtype of avian influenza in Khartoum and Gezira States was reported by the OIE on 20 April 2006. The latest studies showed and indicated widespread of avian influenza type A antibodies in different localities in Sudan (Wegdan et al., 2007).

1.3 Characteristic of Influenza viruses:

Influenza virions are pleomorphic, often spherical but predominantly filamentous in fresh isolates, 80-120 nm in their smallest dimension (Hoyle, 1968, Murphy et al., 1999). Despite their distinctive shape, the filamentous virions possess many of the serological, hemagglutinating, and enzymatic characteristics of the spherical particles. The morphology of influenza virions seems to be primarily determined by the M gene (Bourmakina and Garcia-Sastre ,2003; Roberts and Compans ,1998), although both the HA and NP genes are likely contribute (Jin, Leser, Zhang and Lamb. 1997; Mitnaul, Castrucci, Murti, and Kawaoka. 1996 ; Smirnov, Kuznetsova, and Kaverin. 1991).

Influenza A and B viruses are enveloped viruses with a segmented genome made of eight single-stranded negative RNA segments of 890 to 2,341 nucleotides each (Gürtler, 2006). The A and B types are

distinguished by two integral membrane glycoproteins, HA and NA, that protrude from the virion surface. A third transmembrane protein, encoded by the M2 gene of influenza A viruses and the BM2 gene of influenza B viruses, serves as an ion channel, and within the lipid envelope exists the matrix (M1) protein. The RNA segments are associated with nucleoprotein (NP) and three large polymerase proteins, designated PA, PB1,and PB2 on the basis of their overall acidic or basic amino acid composition, that are responsible for RNA replication and transcription (Krug, Alonso-Caplen, Julkunen and Katze. 1989). The HA spikes are rod-shaped, whereas the NA spikes resemble mushrooms with slender stalks. They are not distinguishable by electron microscopy unless either the HA or NA is removed from the virion surface with a protease or rosettes of the HA and NA are formed after virions are treated with detergent. The NA distribution on virions remains uncertain, but if the HA is removed with trypsin, the NA seems to be evenly distributed (Erickson and Kilbourne, 1980). However, by immunoelectron microscopy with monoclonal antibodies, but not polyclonal antibodies, it seems to be clustered (Amano, Uemoto, Kuroda and Hosaka, 1992; Murti and Webster, 1986).

1.4 Virion properties:

Studies on influenza viruses have long been in the forefront of work on virus structure. The influenza virus was one of the first viruses to be studied in the electron microscope (Taylor, Sharp, Beard, Beard, Dingle, and Feller. 1943), and it demonstrated that certain viruses were assembled by budding from cell membrane (Murphy and Bang, 1952).

1.4.1 Biological properties:

Hirst (1941) and McClelland and Hare (1941) discovered that influenza virus particles agglutinate the erythrocytes of fowl as well as of other animal species.This advance established the concept of

hemagglutinin inhibition in the detection of specific serum antibodies, making it possible to distinguish between viruses of the same type. Hirst (1950) further demonstrated the presence of a receptor-destroying enzyme, now known as neuraminidase. Later biochemical work (Gottschalk, 1957) revealed that influenza viruses contain the HA and NA as major structural and antigenic components of the virus particle.

Influenza A and B virus HAs bind to oligosaccharide containing terminal sialic acids (Weis, Brown, Cusack, Paulson, Skehel and Wiley. 1988**).** Topologically, the binding site is a depression and the amino acid residues that contact the terminal sialic acids , are highly conserved among the different HA subtypes (Nobusawa, Aoyama, Kato, Suzuki, Tateno and Nakajima .1991). The receptor specificity of the HA differs among influenza A viruses. Most avian and equine influenza viruses bind preferentially the NeuAcα2, 3Gal linkage, whereas human and classic H1N1 swine influenza viruses preferentially bind the NeuAcα2, 6Gal linkage on the cell surface sialyloligosaccharides (Rogers and Paulson. 1983**;** Rogers, Pritchett, Lane and Paulson**.** 1983**;** Rogers and D'Souza .1989).

However, an avian H5N1 virus isolated from an individual in Hong Kong in 1997 bound to NeuAcα2, 3Gal-containing receptors but not to those containing an NeuAcα2, 6Gal linkage (Matrosovich, Zhou, Kawaoka and Webster. 1999), indicating that receptor specificity, while being an important determinant of host range restriction, does not prohibit avian-to-human transmission.

1.4.2 Physical properties:

Influenza viruses can remain infectious after 24 to 48 hours on nonporous environmental surfaces and less than 12 hours on porous surfaces (Bean, 1982). Influenza A viruses can persist for extended periods of time in water (WHO, 2006). One study of subtype H3N6 found

that virus resuspended in Mississippi River water was detected for up to 32 days at 4°C and was undetectable after 4 days at 22°C (Webster, 1978). Another study found that several avian influenza viruses persisted in distilled water for 207 days at 17°C and 102 days at 28°C (Stallknecht, 1990). Recent data from studies of H5N1 in domestic ducks have shown that H5N1 can survive in the environment for 6 days at 37ºC (WHO, 2004).

Inactivation of the virus occurs under the following conditions: temperatures of 56ºC for 3 hours, 60ºC, or more, for 30 minutes, acidic pH conditions, presence of oxidizing agents such as sodium dodecyl sulfate, lipid solvents, β-propiolactone and exposure to disinfectants such as formalin and iodine compounds(OIE, 2002).

1.4.3 Chemical properties

The chemical composition of influenza virions can not be given with absolute precision because of the heterogeneity of the virus population and the fact that the composition of the virions is part dependent on the host cell with regard to lipids and carbohydrates. Approximate composition; however, have been determined as follow,

0.8 -1.1% ribonucleic acid (RNA), 70-75% protein, 20-24% lipids and carbohydrates (Ada and Perry, 1954; Frommhagen, Knight and Freeman. 1959; Blough, Weinstien, Lawson, and Kodicek. 1967). The exact molecular weight of influenza virions is also uncertain due to the heterogeneity of the population, and estimations have varied widely. On the basis of electron microscope and sedimentation properties, estimates of $270x10^{n}$ to $290x10^{n}$,(n =6), Daltons have been obtained (Lauffer and Stanley, 1944; Sharp, Taylor, Mclean, Beard and Beard. 1945; Schramm, 1954).

1.4.4 Replication of influenza A virus:

The replication cycle of influenza viruses has been studied most extensively with type A strain. Influenza A virus infects cells through binding of it's HA protein to the cell's sialyloligosaccharide receptor. After binding, the attached virion undergoes endocytosis (White, Kartenbeck and Helenius. 1982). The low pH of the late endocytotic vesicle triggers a conformational change, in the cleavage-activated HA, initiating fusion of the viral and vesicular membranes (Skehel, Bayley, Brown, Martin, Waterfield, White, Wilson and Wiley. 1982). Fusion releases the contents of the virion into the cytoplasm of the cell (uncoating). Before fusion, M2 proteins (BM2 protein for type B viruses, and possibly NB for type B viruses), by ion channeling, introduce protons into the inside of the virion, exposing the core to low pH (Hay, 1992; Helenius, 1992; Pinto, Holsinger and Lamb. 1992; Sugrue, Belshe, and Hay. 1990). Such an event is thought to promote dissociation of the M1 from the RNP by disrupting the low pH-sensitive interaction between these molecules (Zhirnov, 1990), allowing the RNP to migrate to the nucleus through the nuclear pore in an ATP-dependent manner (Kemler, Whittaker and Helenius. 1994; Martin and Helenius, 1991). Once the RNP migrates into the host-cell nucleus, the associated polymerase complexes begin primary transcription of mRNA (Krug, Broni and Bouloy. 1979), which requires cooperation with ongoing transcription by cellular RNA polymerase II. The process of mRNA synthesis begins with incorporation of a G residue complementary to the penultimate C residue on the vRNAs (Beaton and Krug, 1981; Kawakami and Ishihama, 1983). Chain elongation is carried out by the PB1 subunit, which contains four consensus motifs for nucleic acid polymerases (Argos, 1988; Biswas and Nayak ,1994; Ishihama and Barbier ,1994; Poch, Sauvaget, Delarue and Tordo. 1989) as well as nucleotide binding sites (Asano, Mizumoto,

Maruyama, and Ishihama. 1995; Asano and Ishihama, 1997; Romanos and Hay, 1984).

Transcription continues until it reaches the poly (A) addition site, located 15–22 nt from the 5' end of the vRNA. Three elements are critical for polyadenylation: first, is a stretch of uridines near the 5' end of the virion RNA (optimal with 5–7 residues and 16 nt from the 5' end), which serves as the poly (A) signal (Luo, Luytjes, Enami and Palese. 1991 ; Li and Palese, 1994; Poon, Pritlove, Fodor and Brownlee. 1999 ; Zheng , Lee, Palese, García-Sastre .1999), secondly, the RNA duplex of the promoter structure, and third are specific nucleotides near the 5' end of the vRNA (Pritlove, Poon, Devenish, Leahy and Brownlee. 1999). The primary transcripts are then used in the production of viral proteins by the cell's cytoplasmic translation machinery. Three polymerase proteins which are: PA, PB1, and PB2, as well as NP, NS1, and NS2 proteins, are transported to the nucleus. The negative-strand genomic segment and the positive-strand antigenomic segments, but not viral mRNA, are coated with NP. NS1 inhibits the transport of cellular mRNA to the cytoplasm by binding to the poly (A) region (Qiu and Krug, 1994) and maximizing the availability of capped primers for viral mRNA synthesis. The products of viral mRNA synthesis change as infection proceeds which indicate a temporal form of regulation (Hay, Lomniczi, Bellamy and Skehel.1977). Early in infection, the synthesis of mRNAs encoding NP and NS1 dominates; later the production of mRNAs for HA, NA, and M1 increases, while transcripts for the polymerase proteins are relatively low throughout the infection cycle, except at the earliest time (Hatada, Hasegawa, Mukaigawa, Shimizu and Fukuda. 1989). The relative amounts of mRNAs correlate with the amounts of their corresponding proteins, indicating that viral gene expression is regulated at the transcriptional level, in addition to the temporal control mechanisms

governing the translational efficiency of viral mRNA (Yamanaka, Ishihama and Nagata. 1988; Yamanaka, Nagata and Ishihama. 1991). The production of full-length viral complementary RNA (cRNA) was believed to be delayed until viral protein had been synthesized. In this model, newly synthesized NP promoted 'read-through' of the poly (A) site, allowing the synthesis of cRNA transcripts (Beaton and Krug, 1986; Shapiro and Krug, 1988) which in turn serve as templates for the production of vRNA. Recent findings, however, suggest that cRNA synthesis occurs early in infection as well, at this time point, cRNA may be degraded rapidly by cellular nucleases, while increasing amounts of polymerase proteins may protect the cRNA later in infection (Vreede, Jung and Brownlee. 2004). Equimolar quantities of cRNA are synthesized throughout infection (Hay et al., 1977), indicating a lack of regulation of this process.

1.5 Classification of Influenza viruses:

The Orthomyxoviridae family consists of five genera:

Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus, which includes the Thogoto virus and Dhori virus, and Isavirus, which includes infectious salmon anemia virus (ISAV) (Klenk, Cox, Lamb, Mahy, Nakamura, Nuttall, Palese and Rott. 1995) Orthomyxoviruses (Greek: orthos, straight or correct; myxa, mucus) contain segmented, linear, and negative-sense (complementary to mRNA) single-stranded RNA. The number of RNA segments differs among the genera: eight for influenza A, B and ISAV, seven for influenza C, six for Thogoto virus, and probably seven for Dhori virus. Accordingly, influenza A and B viruses contain HA and NA activities in different glycoproteins, whereas influenza C viruses lack NA, containing instead a hemagglutinin–esterase fusion (HEF) protein (Van Regenmortel, 2000).

Currently, there are 16 recognized HA subtypes (Fouchier, Munster, Wallensten, Bestebroer, Herfst, and Smith. 2005), (H1, H2, etc.) and nine NA subtypes (N1, N2, etc.). The full nomenclature for each new isolate includes the type of virus, the host of origin (except for human), geographical site of isolation, strain number, and year of isolation (WHO Memorandum, 1980).

1.5.1 H5N1:

In May 1997, an avian influenza A H5N1 virus infection resulted in the death of a 3-year-old child in Hong Kong. The child died from complications of influenza-associated pneumonia, including acute respiratory distress syndrome, Reye's syndrome, and multiorgan failure. (Rowe, Abernathy, Jean Hu-Primmer, Thompson, Lu, Lim, Fukuda, Cox and Katz. 1999**)**.

Recent genetic characterization of H5N1 strains involved in the current panzootic has demonstrated two distinct phylogenetic clades (Webster, Peiris, Chen and Guan. 2006). Clade **1** viruses have circulated primarily in Cambodia, Thailand, and Vietnam, and clade **2** viruses have circulated primarily in China and Indonesia and have spread westward to the Middle East, Europe, and Africa. Six different subclades of clade 2 have been recognized; three of these are primarily responsible for recent human H5N1 cases. The most recent wave of outbreaks in Thailand (in July 2006) has been caused by viruses closely related to those have caused outbreaks in Thailand in 2004-2005 and to viruses recently circulating in southeast China (Chutinimitkul, Songserm, Amonsin, Payungporn, Suwannakarn, Damrongwatanapokin, Chaisingh, Nuansrichay, Theamboonlers and Poovorawan. 2007) .

AI infections in birds are generally benign, and these viruses are often classified as low pathogenic. However, viruses of the H5 and H7

subtypes are prone to develop into variants that are highly pathogenic for poultry (Alexander, 2000).

A less common mechanism of generation of HPAI viruses has been observed in H7 viruses , which incorporate amino acid sequences derived from theNP or M genes through recombination(Hirst, Caroline, Malachi, Coughlin, Moksa, Zeng, Smailus, Holt, Jones, Marra, Petric, Krajden, Lawrence, Annie, Chow, Skowronski, Tweed, Goh, Robert, Brunham, Robinson, Victoria, Sojonky, Sean Byrne, Li, Kobasa, Booth and Paetzel. 2004 ; Suarez, Senne, Banks, Brown, Essen, Lee, Manvell, Mathieu-Benson, Moreno, Pedersen, Panigrahy, Rojas, Spackman and Alexander. 2004). Regardless of the mechanism of mutation, the result is a virus that spreads cell to cell, causing a systemic infection in birds.

Before 1997, it was believed that molecular constraints restricted the host range of HPAI viruses to infection of avian species. Since the description of HPAI virus infection in 1878 as “fowl plague,” there has never been any indication that the illness could be transmitted to humans, despite the multiple opportunities that have existed through the numerous outbreaks recorded in history. For example, both low and highly pathogenic H5 viruses have been circulating in Mexico since 1993 (Lee, Senne and Suarez. 2004) but no case of human infection with H5 AI was documented there. Although serologic studies in the United States and Europe provided evidence of human infections with various HA subtypes of avian influenza from poultry, these infections were generally subclinical. (Shortridge, 1992).

The H5N1 virus that was first shown to give rise to strains that can transmit to humans appears to have emerged in Southeast China. During 1996, an AI outbreak was observed in geese in Guandong Province The virus was identified as the H5N1 virus and was considered to be an HPAI by virtue of the cleavability of the HA and animal inoculation

studies(Guo, Xu And Wan. 1998). The new H5N1 virus was transported to Hong Kong, where it caused a major HPAI outbreak in chickens and other birds and unexpectedly transmitted to humans (Claas et al., 1998, Subbarao et al., 1998). Human cases of H5N1 have been reported and confirmed in Vietnam, Thailand, Cambodia, and Indonesia. (WHO, 2005a).

Sustained human to human transmission has not been confirmed, but a probable case of limited transmission within a family was recently described in Thailand (Ungchusak, Auewarakul, Dowell, Kitphati, Auwanit, Puthavathana, Uiprasertkul, Boonnak, Pittayawonganon, Cox, Zaki , Thawatsupha, Chittaganpitch, Khontong, Simmerman and Chunsutthiwat. 2005). Experimental data found that cats are readily susceptible, transmit and die of infection with some H5N1 strains (Enserink and Kaiser 2004; Kuiken, Rimmelzwaan, van Riel, van Amerongen, Baars, Fouchier and Osterhaus. 2004). This novel finding contrasts with previous reports that cats could be experimentally infected with human viruses without becoming ill or shedding the virus (Hinshaw, Webster, Easterday and Bean. 1981). More recently, an outbreak of H5N1 in tigers, with evidence of natural tiger-to-tiger transmission, was reported (Thanawongnuwech, Amonsin, Tantilertcharoen, Damrongwatanapokin, Theamboonlers, Payungporn, Nanthapornphiphat, Ratanamungklanon, Tunak, Songserm, Vivatthanavanich, Lekdumrongsak, Kesdangsakonwut, Tunhikorn and Poovorawan. 2005). Although not considered a major threat for transmission of H5N1 to humans, the infections of feline species highlighted our limited understanding of interspecies transmission.

Analysis of the NA sequences found a deletion of 20 amino acids in the stalk region of the molecule, which is commonly found in viruses adapted to terrestrial birds. Further analysis revealed that, since 1997, the NA

gene has been replaced at least 3 times by phylogenetically distinct N1 genes through constant reassortment, suggesting that other viral subtypes contribute to the perpetuation of the H5 viruses in Asia(Guan, Peiris, Lipatov, Ellis, Dyrting, Krauss, Zhang, Webster and Shortridge. 2002; Guan, Poon, Cheung, Ellis, Lim, Lipatov, Chan, Sturm-Ramirez, Cheung, Leung , Yuen, Webster and Peiris. 2004).

1.5.2 H7N3:

This subtype of avian influenza viruses was firstly isolated in 1963 in England where it caused infection for about 29,000 breeder turkeys and accordingly it was named as follow- A/turkey/England/63 (H7N3), also in 1992 it was isolated again in Australia from approximately 12,700 broiler breeders and 5,700 ducks .And in 2004 in Canada- A/chicken/Canada-BC/ 2004(H7N3), caused infection to 53 flocks and 17 million chickens leeding outbreaks with significant spread to numerous farms, resulting in great economic losses while most other outbreaks were associated with only restricted or no spread from the index farms(Alexander ,2000; Kamps, Hoffmann and Wolfgang. 2006**).**

Although all virulent strains, of avian influenza, isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. Due to the risk of a low virulent H5 or H7 becoming virulent by mutation in poultry hosts, all H5 and H7 viruses have also been classified as Notifiable Avian Influenza (NAI) viruses (OIE, 2005).

1.6 Mutation of Influenza virus:

The epidemiological success of influenza viruses is largely due to the two types of antigenic variation that occur in the HA and the NA. Antigenic variations in HA and NA proteins have resulted in the classification of type A influenza into subtypes (Wharton, Weis, Skehel, and Wiley. 1989), and respectively renders an individual susceptible to

infection with new strains , despite previous experience with other influenza viruses.

The first kind of variation, called antigenic drift, occurs with both influenza A and B viruses, and it is the gradual alteration of the structure of the protein by single amino acid substitutions (mutation) in the HA and NA within type B influenza viruses or within a given subtype of influenza A viruses. This kind of variation is the result of positive selection of spontaneous mutants by neutralizing antibodies (Bush, Bender, Subbarao, Cox and Walter. 1999; Plotkin and Dushoff, 2003). Recent phylogenetic analysis have also shown that, although co-circulating lineages of HA genes of influenza B viruses can co-exist longer than for influenza A hemagglutinin genes, the overall patterns and the rates of evolution of the HA genes of these two types of influenza viruses are more similar than was previously believed (Cox and Bender, 1995).

Only influenza A viruses exhibit the second, more dramatic kind of antigenic variation which called antigenic shift. Antigenic shift is the appearance of a new subtypes of influenza A viruses containing a novel HA, or a novel HA and NA, immunologically distinct from isolates circulating previously. When antigenic shift occurs, the HA of the new strain would be expected to vary at the amino acid level 20–50 percent from the corresponding protein of previously circulating strains. Antigenic shift is responsible for worldwide pandemics, which occur at irregular and unpredictable intervals.

Although 16 subtypes of influenza HA have been identified in avian species, epidemics and pandemics of influenza among humans during this century seem to have been caused by viruses with HAs of only three different subtypes: H1, H2, and H3. While the pandemic human influenza viruses of 1957 (H2N2) and 1968 (H3N2) clearly arose through reassortment between human and avian viruses, the influenza virus

caused the Spanish flu in 1918 ,which was the most severe recorded pandemic of influenza, appears to be entirely derived from an avian source (Reid, Taubenberger and Fanning. 2004; Belshe, 2005).

1.7 Transmission:

Human influenza is transmitted by inhalation of infectious droplets and droplet nuclei by direct contact, and perhaps, by indirect (fomite) contact, with self-inoculation onto the upper respiratory tract or conjunctival mucosa. **(**Salgado, Farr, Hall and Hayden. 2002).

The relative efficiency of the different routes of transmission has not been defined. For human influenza A (H5N1) infections, evidence is consistent with bird-to-human, possibly environment-to-human, and limited, non sustained human-to-human transmission to date (Bridges, Kuehnert and Hall. 2003).

1.7.1 Animal to Human:

In 1997, exposure to live poultry within a week before the onset of illness was associated with disease in humans, whereas there was no significant risk related to eating or preparing poultry products or exposure to persons with influenza A (H5N1) disease. (Mounts, Kwong, Izurieta, Ho, Au, Lee, Buxton, Williams, Mak, Katz, Thompson, Cox and Fukuda. 1997). Exposure to ill poultry and butchering of birds were associated with seropositivity for influenza A (H5N1) (Bridges, Lim, Hu-Primmer, Sims, Mak, Fukuda, Rowe, Thompson, Conn, Lu, Cox and Katz. 2002).

1.7.2 Human to Human:

Human-to-human transmission of influenza A (H5N1) has been suggested in several household clusters and in one case of apparent child-to-mother transmission (Ungchusak et al., 2005). Intimate contact without the use of precautions was implicated, and so far no case of human-to-human transmission of H5N1 virus by small-particle aerosols has been

identified. In 1997, human-to-human transmission did not apparently occur through social contact, (Katz, Lim, Bridges, Rowe, Hu-Primmer, Lu, Abernathy, Clarke, Conn, Izurieta, Kwong, Mak and Cox. 1999), and serologic studies of exposed health care workers indicated that transmission was inefficient (Bridges, Katz, Seto, Chan, Tsang, Ho, Mak, Lim, Tam, Clarke, Williams, Mounts, Bresee, Conn, Rowe, Hu, Abernathy, Lu, Cox, and Fukuda. 2000).

1.7.3 Environment to Human:

Given the survival of influenza A (H5N1) in the environment, several other modes of transmission are theoretically possible. Oral ingestion of contaminated water during swimming and direct intranasal or conjunctival inoculation during exposure to water are other potential modes, as is contamination of hands from infected fomites and subsequent self-inoculation. The widespread use of untreated poultry feces as fertilizer is another possible risk factor (WHO. 2005b).

1.8 Clinical features:

The clinical spectrum of influenza A (H5N1) in humans is based on descriptions of hospitalized patients. The frequencies of milder illnesses, sub clinical infections, and a typical presentations (e.g., encephalopathy and gastroenteritis) have not been determined, but case reports indicate that each occurs. Most patients have been previously healthy young children or adults. (Apisarnthanarak, Kitphati, Thongphubeth, Patoomanunt, Anthanont, Auwanit, Thawatsupha, Chittaganpitch, Saeng-Aroon, Waicharoen, Apisarnthanarak, Storch, Mundy and Fraser. 2004; WHO. 2005c; De Jong, Cam, Qui, Hien, Thanh, Hue, Beld, Phuong, Khanh, Chau, Hien, Ha and Farrar. 2005).

1.8.1. Incubation:

The incubation period of avian influenza A (H5N1) may be longer than for other known human influenzas. In 1997, most cases occurred within two to four days after exposure (Yuen, Chan, Piris, Tsang, Que, and Shortridge, 1998); recent reports indicate similar intervals but with ranges of up to eight days. The case-to-case intervals in household clusters have generally been 2 to 5 days, but the upper limit has been 8 to 17 days, possibly owing to unrecognized exposure to infected animals or environmental sources (Chotpitayasunondh, Ungchusak, Hanshaoworakul, Chunsuthiwat and Sawanpanyalert. 2005 ; Hien, Liem, Dung, San, Mai, Chau, Suu, Dong, Mai, Thi, Khoa, Phat, Troung, Long, Tung, Giang, Tho, Nga, Tien, San, Tuan, Dolecek, Thanh, Jong, Schultsz, Cheng, Lim, Horby and Farrar. 2004).

1.8.2. Initial Symptoms:

Most patients have initial symptoms of high fever (typically a temperature of more than 38°C) and an influenza-like illness with lower respiratory tract symptoms (WHO. 2004). Upper respiratory tract symptoms are present only sometimes. Unlike patients with infections caused by avian influenza A (H7) viruses, patients with avian influenza A (H5N1) rarely have conjunctivitis(Fouchier et al.,2004**)**. Diarrhea, vomiting, abdominal pain, pleuritic pain, and bleeding from the nose and gums have also been reported early in the course of illness in some patients (Chan, 2002; Chotpitayasunondh et al., 2005; Hien et al., 2004; Tam, 2002). Watery diarrhea without blood or inflammatory changes appears to be more common than in influenza due to human viruses (Nicholson, 1998), and may precede respiratory manifestations up to one week (Apisarnthanarak et al., 2004). One report described two patients who presented with an encephalopathic illness and diarrhea without apparent respiratory symptoms (De Jong et al., 2005).

1.8.4. Mortality:

The fatality rate among hospitalized patients has been high, although the overall rate is probably much lower (WHO. 2005c). In contrast to 1997, when most deaths occurred among patients older than 13 years of age, recent avian influenza A (H5N1) infections have caused high rates of death among infants and young children. The case fatality rate was 89 percent among those younger than 15 years of age in Thailand. Death has occurred in an average of 9 or 10 days after the onset of illness, and the range was 6 to 30, and most patients have died of progressive respiratory failure (Chotpitayasunondh et al., 2005; Hien et al., 2004).

1.9 Pathogenicity:

Much remains to be learned about the pathogenesis of influenza virus replication and its relationship to the clinical manifestations and complications of the infection. Many studies to investigate the pathogenesis of influenza infections were conducted during the 1957/8 pandemic of Asian influenza (Hers and Mulder, 1961 ; Walsh, Dietlein, Low, Burch and Mogabgab. 1961). These studies demonstrated that the principal site of replication is the columnar epithelial cells, but histological studies indicate that viral replication can occur throughout the respiratory tract. Infected ciliated columnar cells become vacuolated and lose their cilia, and infected mucosal and ciliated epithelial cells become necrotic and desquamate. Regeneration of the respiratory epithelial cells takes about 3–4 weeks, during which time pulmonary function abnormalities may persist (Hall, Douglas, Hyde, Roth, Cross, and Speers. 1976). In these typical cases of influenza in which infection is confined to the respiratory tract, prostration, fever, and myalgia often seem to be disproportionate to objective clinical signs or observed pathological changes. Lungs from fatal cases of primary viral pneumonia most notably show hyaline membrane coverage of alveolar walls together

with extensive intra-alveolar edema and hemorrhage. Tracheitis and bronchitis are also observed (Hers and Mulder, 1961; Martin, Kunin, Gottlieb, Barnes, Liu and Finland. 1959). The occurrence of systemic illness and fever suggests dissemination of virus via the blood stream, but systematic studies (Kilbourne, 1959; Minuse, and Willis. 1962) and limited case reports (Lehmann and Gust, 1971; Naficy, 1963) suggest that viremia is detected only rarely.

1.10 Diagnosis:

A number of techniques have been developed for the diagnosis of influenza virus infections. Virus isolation in cultured cells or in fertilized hens' eggs or demonstration of a four-fold or greater rise in specific antibodies between acute and convalescent sera are techniques that have been used for many years. More recently, detection of viral antigens directly in clinical specimens by immunological methods and detection of viral nucleic acids by hybridization or using polymerase chain reaction (PCR) has greatly increased the speed of laboratory diagnosis.

1.10.1 Isolation of the viruses:

The historical record of influenza viruses remained sparse until technological advances permitted their isolation. In 1901, a 'filterable agent' was isolated from chickens suffering from fowl plague which was later classified by Schafer (1955) as influenza A virus. Smith, Andrews and Laidlaw (1933), inoculated ferrets intranasally with human nasopharyngeal washes, which produced a form of influenza that spread to the animals' cage mates.

An antigenically distinct virus, isolated by Francis (1940), was classified as a type B strain (B/Lee/40) to distinguish it from the 1933 isolate. The third major type of influenza virus, influenza C, was first isolated in 1947 by Taylor (Taylor, 1949).

Embryonated hens' eggs, a number of primary tissue culture systems and continuous cell lines, such as Madin– Darby canine kidney (MDCK, American Type Culture Collection, Rockville MD), cells can be used to isolate and grow influenza viruses for identification or research purposes. On initial passage in eggs or tissue culture, some influenza viruses preferentially agglutinate guinea-pig over chicken erythrocytes, but on continued passage the viruses may preferentially agglutinate erythrocytes from chickens (Burnet and Bull, 1943).

1.10.2- Isolation in Embryonated Eggs:

Soon after the first identification of human influenza A viruses, Burnet (1936) reported that embryonated hens' eggs could serve as a host system for their propagation. This host system is still used for vaccine production and for generating large quantities of influenza virus that are occasionally necessary for research. To isolate both type A and type B influenza viruses, clinical samples are inoculated into the amniotic and allantoic cavities of 10– 11-day-old embryonated hens' eggs. The eggs are usually incubated at 33˚–34˚_C for 2–3 days before the virus is harvested. Most type A and B influenza viruses that are originally isolated in eggs will grow well in the allantoic cavity after one or two passages. Type C influenza viruses, on the other hand, grow only in the amniotic cavity and are best propagated in 7–8-day-old embryonated hens' eggs after 5 days' incubation (Burnet and Bull, 1943).

1.10.3 Isolation in Tissue Culture:

Primary cynomolgus or rhesus monkey kidney cells (PMKC) are susceptible to a variety of respiratory viruses, including influenza viruses. Disadvantages with these cells include their cost and the presence of spumaviruses. Influenza A, B, and C viruses can also be isolated in the MDCK cell line in the presence of trypsin. Primary monkey kidney and

MDCK cells are most often used for the primary isolation of influenza viruses from humans.

Influenza virus isolation in tissue culture is still used in many laboratories worldwide and when performed correctly, with properly collected specimens along with good quality laboratory cells and reagents, this method is highly sensitive. The shell vial tissue culture isolation method combines rapid detection of virus in the inoculated cells after 48– 72 h, increased sensitivity being obtained by centrifugation of specimens onto the cells. Monoclonal antibodies are often used for immunofluorescent detection of viral antigens in the inoculated cells (Kalin and Grandien, 1993).

1.10.4 Serology:

Serologic testing can be used for retrospective diagnosis of infection but is rarely useful for patient management and is not widely available (Hayden and Palse. 2002; Treanor, 2005). Acute-phase sera should be collected within 1 week after illness onset and convalescent sera should be collected 2 to 3 weeks later.

The most common serologic methods are complement fixation (CF) Agar-gel immunodiffusion (AGID), Hemagglutination inhibition (HI) tests, and enzyme-linked immunosorbent assay (ELISA). A variety of other methods, such as neutralization, microneutralization, single radial hemolysis, radial immunodiffusion, and Western blot, have been reported (Hayden and Palse. 2002; Rowe et al., 1999).

1.10.4.1 Agar gel-immunodiffusion test (AGID):

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens (OIE, 2005). This fact enables the presence or absence of antibodies to any influenza A virus to be detected by AGID test that have been widely and routinely used to detect specific antibodies in serum as an indication of infection. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated fowl eggs that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 **g**. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen (Beard, 1970).

1.10.4.2 Haemagglutination (HA) and Haemagglutination Inhibition (HI) tests:

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples are applied using U-bottomed micro-well plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.1 M), pH 7.0–7.2, and red blood cells (RBCs) taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension (OIE, 2005).

1.10.4.2.1 Haemagglutination (HA) test:

The surface HA glycoprotein of influenza virus isolates will bind to variety of mammalian and avian erythrocytes, and this phenomenon is the basis for screening of 9 days old inoculated allantoic fluids for the presence of agglutinating agents. Hemagglutination is determined by making twofold serial dilution in a microtiter plate followed by the additioin of an equal volume of 1% washed chicken erythrocytes. Diluting the allantoic fluid avoids the occasional prozone phenomenon that can occur with undiluted egg fluids (WHO, 2002; Swayne, Senne and Beard. 1998**).**

1.10.4.2 .2 Haemagglutination Inhibition (HI) test:

The hemagglutination inhibition (HI) assay, the standard method for serologic detection of influenza virus infection in humans, has been shown to be less sensitive for the detection of antibodies induced by avian influenza viruses (Lu, Webster and Hinshaw 1982; Rowe et al., 1999). Because of the instability of HA, the dilution of antigen used must be precisely determined by HA titration each time the HI test is performed. The test is also complicated by the presence in sera of several species of nonspecific inhibitors of hemagglutination(WHO, 2002). These inhibitors interact with the HA molecule and prevent the agglutination of erythrocytes, even in the absence of specific antibodies. The non-specific inhibitors fall into three classes: (α) inhibitors, which are present in human serum and are heat-stable sialylated glycoproteins that inhibit hemagglutination but do not neutralize viral infectivity; (β) inhibitors, which are also present in human serum but are heat-labile and have neutralizing activity; and (γ) inhibitors, which are present in horse serum and are heat stable sialylated sialoproteins with neutralizing activity. A variety of techniques to inactivate serum inhibitors have been developed. These include treatment with heat, receptor-destroying enzyme (RDE) of

Vibrio cholerae, trypsin, periodate, or a combination of these factors (Kendal, Skehel and Pereira. 1982).

1.10.4.3 Enzyme Linked Immunosorbent Assay (ELISA):

Competitive ELISA formats allow the examination of sera of all bird species, independent from the availability of species-specific conjugates (Shafer, Katz, Eernisse. 1998; Zhou, Chan, Heckert and Cantin. 1998). An ELISA format for the detection of H7-specific antibodies has been reported (Sala, Cordioli, Moreno-Martin, Tollis, Brocchi, Piccirillo and Lavazza. 2003).

1.10.4.4 Immunofluorescence:

For direct immunofluorescence, potentially infected respiratory epithelial cells are fixed to a slide and viral antigens contained in the cells is detected by specific antibodies which are either directly conjugated to a fluorescent dye (direct immunofluorescence) or detected by anti-antibodies linked to a fluorescent dye (indirect immunofluorescence). In both cases reactions are visualised under the fluorescence microscope and positive cells are distinguished on colour intensity and morphology of fluorescent areas. Compared to complement fixation and AGID tests, immunofluorescence test was more sensitive and could be used as quantitative method for the detection of antibody to the RNA and surface antigens (Stumpa, Fedova and Tumova. 1975**).**

1.10.4.5 Neuraminidase Inhibition (NI) test:

Influenza virus neuraminidase (NA) enzyme is important for the release of virions from the host cell surface and viral aggregates and may also be involved in ensuring that the virus is targeted to respiratory epithelial cells (Gubareva, Kaiser and Hayden. 2000).

The major steps to perform the test include:

(i) Assays of the test virus for neuraminidase activity.

(ii) Adjustment of virus dosage to a standard unit of neuraminidase activity.

(iii) Assays of the antibodies by testing serial dilution of the tested antiserum for inhibition of standard units of neuraminidase (Aymard, Coleman, Dowdle, Laver, Schild and Webster. 1973).

The neuraminidase inhibition test is performed by incubating the standard viral neuraminidase dose with serial dilution of normal and tested sera. The inhibitory effect serum on neuraminidase activity is determined and the neuraminidase inhibition titer is calculated.

1.10.4.6 Complement Fixation (CF) test:

Complement fixation tests are based on the ability of antigen-antibody complexes to consume complement which results in no compliment being available to lyse sensitized sheep red blood cells. These assays are intensive laborious and necessitate controls for each procedure but reagents are cheap and widely available. CF assays are less sensitive than HI both in the diagnosis of acute infection and the determination of immunity after vaccination (Prince and Laber. 2003).

1.10.5 Molecular techniques:

1.10.5.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

Reverse transcriptase-Polymerase chain Reaction (RT-PCR) procedure have been developed to detect the presence of nucleic acid of influenza virus in clinical samples and to identify virulence markers associated with HPAI viruses in subtypes H5 and H7(Horimoto and Kawaoka, 1995). Although detection of nucleic acid by RT-PCR procedures can aid in the presumptive diagnosis of avian influenza, isolation of the virus is required for subtype and pathogenicity determination .Virulence markers in H5 and H7 subtypes of avian influenza viruses have been characterized by the presence of multiple

basic amino acids at the hemagglutinin cleavage site. RT-PCR techniques on clinical specimens could, with the correctly defined primers, result in rapid detection and subtype (at least of H5 and H7) identification, plus a cDNA product that could be used for nucleotide sequencing **(**Munch, Nielsen, Handberg and Jorgensen. 2001; Starick, Romer-Oberdorfer and Werner. 2000; Suarez, 1998**).**

1.11 Control of Influenza disease:

1.11.1 Vaccination:

Vaccination is currently the most effective measure for reducing the impact of influenza among human or avians. Immunization against human influenza is focused mainly on individuals at increased risk of complications of influenza infection and on individuals who might transmit influenza to such people (Fedson, Hannoun, Leese, Sprenger, Hampson, Bro-Jorgensen, Ahlbom, Nokleby, Valle, Olafsson, Garcia, Gugelman, de Andrade, Snacken, Ambrosch and Isa. 1995; Nicholson, Snacken and Palache. 1995).

Experimental work has shown, for both highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), that vaccination protects against clinical signs and mortality, reduces virus shedding and increases resistance to infection, protects from diverse field viruses within the same hemagglutinin subtype, protects from low and high challenge exposure, and reduces contact transmission of challenge virus (Swayne, 2003; Swayne and Suarez, 2000; Capua, Terregino, Cattoli and Toffan. 2004 ; European Union . 2003).

Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic changes in the virus and this has been reported to have occurred in Mexico (Lee et al., 2004). Conventionally, vaccines that have been used against HPAI or LPAI have been prepared

from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil. Inactivated vaccine was prepared from the LPAI virus of H7N3 subtype responsible for a series of outbreaks in turkeys in Utah in 1995 and used, with other measures, to bring the outbreaks under control (Halvorson, Frame, Friendshuh and Shaw. 1998). Regarding humans, no influenza A (H5) vaccines are currently commercially available for humans. Earlier H5 vaccines were poorly immunogenic and required two doses of high hemagglutinin antigen content (Treanor, Wilkinson, Masseoud, Hu-Primmer, Battaglia, O'Brien, Wolff, Rabinovich, Blackwelder and Katz. 2001), or the addition of MF59 adjuvant (Nicholson, Colegate, Podda, Stephenson, Wood and Zambon. 2001), to generate neutralizing antibody responses. A third injection of adjuvanted 1997 H5 vaccine variably induced cross-reacting antibodies to human isolates from 2004 (Stephenson, Bugarini, Nicholson, Podda, Wood, Zambon and Katz. 2005). Reverse genetics has been used for the rapid generation of nonvirulent vaccine viruses from recent influenza A (H5) isolates, (Webby, Perez, Coleman, Guan, Knight, Govorkova, McClain-Moss, Peiris, Rehg, Tuomanen and Webster. 2004**)**, and several candidate vaccines are under study. One such inactivated vaccine with the use of a human H5N1 isolate from 2004 has been reported to be immunogenic at high hemagglutinin doses (Altman, 2005). Studies with approved adjuvants like alum are urgently needed. Live attenuated, cold-adapted intranasal vaccines are also under development; these are protective against human influenza after a single dose in young children (Belshe, Mendelman, Treanor, King, Gruber, Piedra, Bernstein, Hayden, Kotloff, Zangwill, Iacuzio and Wolff. 1998).

1.12 Treatment:

Antivirals that commonly used in treatment of Influenza A subtypes infection in human are devided into two major groups:

A- M2 Inhibitors:

– Amantidine is only effective against influenza A, and some naturally occurring strains of influenza A are resistant to it, the compound has been shown to have both therapeutic and prophylactic effects.

– Rimantidine is similar to amantidine but has fewer side effects. It is used both for treatment and prophylaxis of influenza A infection in persons one year or older. Amantadine and rimantadine both interfere with the replication cycle of all subtypes of type A but not type B influenza viruses (Hayden ,1996 ; Van Voris and Newell ,1992), they inhibit the replication of influenza A viruses at low, clinically achievable concentrations of <1 μg/ml (Tominack and Hayden ,1987).

B- Neuraminidase inhibitors:

– Zanamivir, the first neuraminidase inhibitor available for clinical use, is effective against both influenza A and B. It must be administered by inhalation. It is used as treatment for influenza A and B in persons 12 years or older but not for prophylaxis.

– Oseltamivir, can be given orally. Shown to be effective and devoid of significant side effects in clinical trials. The first involves inhibition of the acid mediated dissociation of the matrix protein from the RNP complex within endosomes early in replication; this dissociation is essential for initiating viral replication (Pinto et al., 1992; Sugrue et al., 1990). A second effect on virus maturation relating to a low pH-mediated alteration of the HA protein during its transport to the cell surface occurs during the replication of certain avian influenza viruses (Ohuchi, Cramer, Vey, Ohuchi, Garten and Klenk. 1994; Sugrue et al., 1990; Takeuchi and Lamb, 1994).

Viral strains responsible for pandemics and epidemics in recent years have all been drug-sensitive (Hayden, 1996), but resistance to these compounds is readily selected by growth in the presence of these drugs either in vivo or in vitro. Resistant viruses seem to be pathogenic and can cause typical influenza, but resistant viruses are no more transmissible or virulent than strains sensitive to amantadine and rimantadine (Hayden, 1996). Drug resistance develops rapidly in viruses exposed to amantadine or rimantadine, and may emerge during treatment.(Couch ,1996 ; Acha, and Szyfres, 2003 ; Harper, Fukuda, Uyeki, Cox and Bridges. 2004). Some of the H5N1 viruses isolated in 2004 in Asia have been resistant to amantadine and rimantadine. Laboratory studies have shown that influenza viruses can also become resistant to zanamivir and oseltamivir (Harper et al., 2004).

Patients with suspected influenza A (H5N1) should promptly receive a neuraminidase inhibitor pending the results of diagnostic laboratory testing, but optimal dose and duration of treatment with neuraminidase inhibitors are uncertain, and currently approved regimens likely represent the minimum required. These viruses are susceptible in vitro to oseltamivir and zanamivir. (Leneva, Roberts, Govorkova, Goloubeva and Webster. 2000; Govorkova, Leneva, Goloubeva, Bush and Webster. 2001). In contrast two isolates from the 1997 outbreak, recent human influenza A (H5N1) isolates are highly resistant to the M2 inhibitors, amantadine and rimantadine, and consequently, these drugs do not have a therapeutic role. Agents of clinical investigational interest for treatment include zanamivir, peramivir, long-acting topical neuraminidase inhibitors, ribavirin (Madren, Shipman and Hayden. 1995, Knight and Gilbert, 1987), and possibly, interferon alfa. (Baron and Isaacs, 1962).

In recent years, WHO and the governments of many countries have been stockpiling antiviral drugs, primarily Tamiflu, for prophylaxis and treatment(Longini, Nizam, Xu, Ungchusak, Hanshaoworakul, Cummings and Elizabeth. 2005). Tamiflu is a popular choice for stockpiling, since it is available in both pill form and a drinkable liquid suspension (Normile, 2006a). Supply of Tamiflu will be boosted further because a handful of generic drug makers have started producing their own version of Tamiflu with a sub-license from Roche Laboratories Inc (Normile, 2006b). For example, Roche Laboratories Inc. has granted sub-licenses to companies in China and India to manufacture Tamiflu for pandemic use.

In November 2006, the US Food and Drug Administration revised the product labeling requirement of Tamiflu to include new warnings about the possibility of self-injury and delirium associated with the drug (Normile, 2006c).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Serum sample collection:

Two hundred and twenty two (222) serum samples were collected randomly in March 2007, from patients with no apparent clinical signs of influenza or other influenza like disease, in the laboratories of the Sudan Atomic Energy Commission (SAEC) in Khartoum. One venous sample of blood was taken from each patient by laboratory technicians in 5mL syringe and serum was left to separate. Extra separation process of sera was done in the laboratory by centrifugation which was followed by allocating of each serum sample into three different Eppindorff tubes. The details of collected samples are demonstrated in tables 1, 2 and 3 below.

Table 1: Frequency of collected human serum Samples by Gender.

Gender	No. of collected	Percent
Male	43	19.4
Female	179	80.6
Total	222	100.0

Table 2: Frequency of collected human serum Samples by Age groups.

Age group	Frequency	Percent
Less than 20	12	5.4
20 - 30	85	38.3
31 - 40	64	28.8
41 - 50	35	15.8
51 - 60	22	9.9
More than 60	4	1.8
Total	222	100.0

Table 3: Frequency of collected human serum Samples by Location.

Location	Frequency	Percent
Khartoum	141	63.5
Omdurman	81	36.5
Total	222	100.0

2.2- Laboratory Work:

2.2.1-Preparation and Sterilization of Glassware:

Pipettes, Petri dishes, pestles and mortars, sand, test tubes, forceps , scalpels and scissors , were sterilized in hot oven at 160˚C for two hours.

Tips, Eppendorff tubes, bottles with rubber caps and buffer solutions were autoclaved at 120˚C for 15 minutes. Microtiter plates were washed with 1% NaOH to remove the traces of the RBCs completely and then they were put overnight in the same solution. The next day they were rinsed with tap water and kept in 1% HCl solution for two hours. Finally they were washed in four changes of deionized distilled water and left to dry at room temperature. At every test they were swabbed with a piece of cotton and phosphate buffer saline (PBS) solution.

2.2.2-Avian Influenza Antigens:

Reference strains of H5N1/Mallad/Neth/12/00 and H7N3/Mallad/ Neth/12/00 were donated by WHO Reference Laboratory in the United Kingdom.

2.2.3- Reference antisera:

Reference antiserum of the H5N/Mallad/Neth/12/00/Rabbit anti-sera, and H7N3/Mallad/Neth/12/00/Goat, antisera were also donated by WHO Reference Laboratory in the United Kingdom.

2.2.4 Virus Propagation and Identification:

2.2.4.1 Embryonated Egg Inoculation and Harvest:

2.2.4.1.1 Inoculation of the Chorioallantoic Membrane (CAM):

A central spot that just at the top of the air cell was marked on a 13 days old embryonated egg then a hole was made just to penetrate the shell and shell membrane. Another hole was made in an area that is free of blood vessels and by the side of the egg. The dropping of the membrane was verified in dark room by candling, then a rubber bulb

was placed over the hole in the air cell to slowly aspirate air from the cell by releasing pressure on the deflated bulb, the suction caused false air cell to form in the area of the second hole. To deliver the inoculums on the chorioallantoic membrane (CAM), the tip of 1ml insulin syringe needle was inserted just within the shell and 0.2ml was inoculated into each embryonated eggs. The eggs were incubated at 37ºC, and were examined daily for 5 days.

2.2.4.1.2 Harvest of Chorioallantoic Membranes (CAM):

After 3-6 days of incubation the shell was disinfected on the small end of the egg by 70% alcohol, and the end of the egg was cracked off by sterile forceps. The CAM was removed from the shell by sterile forceps. The presence of the virus was detected by the appearance of thickness and or hemorrhages of CAMs, which were then moved to sterile mortar and grounded carefully with the aid of sterile sand and PBS using a pestle. The suspension was then subjected to three times series of freezing and thawing followed by centrifugation at 1000/rpm for 10 minutes. The supernatant was then collected and checked for hemagglutination activity.

2.2.4.1.3 Inoculation of Allantoic Cavity:

The embryonated eggs, which inoculated, were 9 days old that candled to determine the edge of the air sac. A spot was marked, using a pencil, right in the opposite side of the air sac and about 2-4mm far from the edge. A cotton wool and 70 percent alcohol were used to swab the end of the inoculated eggs, and the alcohol was allowed to evaporate. A hole was pierced horizontally at the end of the embryonated egg at the marked inoculation spot. One mL insulin syringe was used to inoculate about 0.2 mL of the virus suspension, vertically, through the hole in the eggshell, into the allantoic cavity. The needle was withdrawn from the egg and the hole was sealed with melted wax. The inoculated eggs were incubated at 37˚C and checked by candling against light daily for five

days. Embryos that died within 24 hour were discarded and the survivals were transferred to refrigerator and chilled at 4˚C overnight and then were harvested.

2.2.4.1.4 Harvest of Allantoic Fluids:

The top of each harvested embryonated egg was cleaned with 70% ethanol, and sterile forceps was used to break the egg shell over the air sac. Then the chorioallantoic membrane was punctured, and the allantoic membrane was pushed a side by the forceps. A rapid hemagglutination test was performed in a clean ceramic plate to check the presence of agglutinating agent. The allantoic fluid was then aspirated using 5ml syringe and transferred to sterile screw capped tube. The harvested fluid were then centrifuged at 1000/rpm for 5 minutes and stored at -20˚C.

2.2.5 Hemagglutination and Hemagglutination Inhibition:

2.2.5.1 Preparation of 1% RBCs:

Blood was collected from the wing vein from a minimum of three healthy chickens in an equal volume of Alsever's solution using 5mL disposable syringe. The red blood cells (RBCs) were then transferred to a sterile tube and washed three times with PBS solution by centrifuging at 1000 rpm for about 5 minutes. After the last wash the RBCs pellet was collected and measured to prepare 1% suspension by diluting with PBS solution.

2.2.5.2-Hemagglutination test:

Microtiter plates with U-shaped bottoms and washed RBCs were used to determine the titer of the two viruses (H5N1, H7N3) each at it's time.

A volume of 50µL of PBS (pH 7.2) was added to all wells using a multichannel micropipette. Then the antigen was added to the first well in each row except the last two, which were filled with 100 µL as

control negative wells. A serial twofold dilution was then made by transferring 50 µL from the first well, that containing the virus to the next well and the last 50 µL were discarded.

Fifty microliter of 1% RBCs suspension were then added to these wells and mixed manually. The plates were incubated for 30 minutes at room temperature. The virus titer is considered the reciprocal of the last dilution that gave a complete hemagglutination.

2.2.5.3 Hemagglutination Inhibition test:

This test was done to insure that the antigens used are specifically H5 and H7. Serial twofold dilutions of the reference antiserum to the two viruses (H5N1 and H7N3) were done in U-shaped micro titter plate in PBS solution. Then equal volumes of four hemagglutinating units (4HAU) were added and after incubation at room temperature for 30 minutes RBCs were added and incubated again for 40 minutes.

2.2.6 Serological tests:

2.2.6.1 Agar gel-immunodiffusion test (AGID):

2.2.6.1.2 Preparation of antigen for the AGID test:

This test was done basically according to the method that used by Beard (1970) with some modification from OIE (2005).

Preparations of nucleocapsid-rich antigen were obtained from chorioallantoic membranes for use in the AGID test. This method involves removal of the chorioallantoic membranes that collected previously, from infected embryonated eggs. The membranes are then homogenised or ground to a paste using pestle and mortar after the addition of suitable amount of phosphate buffer saline (PBS), with pH 7.2 in intervals. This is subjected to three freeze-thaw cycles, followed by centrifugation at 1000 **g** for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin.

2.2.6.1.3 Preparation of agarose:

The method used is similar to that used by Beard (1970).

A weight of 1 gram agarose and 0.8 grams of Sodium chloride (NaCl) were added to 100ml of deionized distilled water (D.D.W) in 1 liter sterile glass flask. The mixture was then boiled in microwave for two minutes until it became clear. A volume of 0.5ml phenol was added as preservative.

2.2.6.1.4 Performing the AGID test:

2.2.6.1.4.1 Preparation of Plates:

About 20ml of liquefied agarose were dispensed in 100x15 mm Petri dishes to create about 2.8~3 mm thickness. The plates were left to cool on a leveling table in dust free environment at room temperature. After the agarose was hardened, the wells were cut out using a template and the diameter of each well was 5.0 mm, and the distance between the central and peripheral wells was 2.4 mm. Five patterns were cut in each plate and the agarose plugs were removed manually. Wells were sealed by drops of agarose as control step.

2.2.6.1.4.2 Method for examination of samples with AGID:

Equal volumes of influenza A (H5N1) AGID antigen, that was prepared before, and 1% Sodium Dodecyl Sulphate (SDS) were mixed together to enhance the diffusion of the antigen. Then 50 µL from the mixture were transfered to the central wells of each pattern, using a micropipette, and a 50 µL of positive control serum was placed in one of the peripheral wells in each rosette as same as negative controls. The remaining wells were filled with the tested sera.

The plates were then covered and incubated in humid chamber at room temperature and examined daily, against light and dark background, for precipitation lines at least for 48 hours. Positive results were recorded and

plates were stained by Comassi Brilliant Blue stain, to improve visualization.

2.2.6.1.4.3 Interpretation of the results:

Precipitin lines were detected after approximately 48 hours and observed using illuminator with a dark background from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the tested serum well. Crossed lines are interpreted to be due to the test serum lacking identity with the antibodies in the positive control well.

2.2.6.2 Hemagglutination Inhibition test:

2.2.6.2.1 Preparation of 4HAU of the virus:

Microtiter plates with U-shaped bottoms and 1% washed chicken RBCs were used to determine the four hemagglutinating units (4HAU) of the antigens each time the test was performed.

A fifty µL of PBS (pH 7.2) were dispensed in all of the plate wells, followed by the addition of 50 µL from the antigen to the first well in each row except rows G and H which were filled with 100 µL of the RBCs as control wells. A serial twofold dilutions were made by transferring 50 µL from the first wells, which containing the antigens, using multichannel micropipette to the successive columns, and the last 50 µL were discarded. Then 50 µL of the RBCs suspension were added to each well, except control wells, and mixed by shaking manually. The plates were then incubated at room temperature for half an hour, and then were read.

The highest dilution of the virus which caused complete hemagglutination is considered the titration end point, and the titer was considered the reciprocal of the virus dilution in the last well with complete hemagglutination which also considered to be containing one

hemagglutinating unit (1HAU). The dilution that containing 4HAU was then calculated.

2.2.6.2.2 Pre-treatment of sera to remove non-specific inhibitors:

One sample of serum was collected from people without obvious clinical signs of influenza or other influenza like diseases. Treatment of sera with trypsin and heat was found to be one of the best methods for removing non-specific inhibitors in human sera (Sampaio and Isaacs 1953). The trypsin solution was prepared by dilution of 10x2.5% concentrated trypsin to 0.04% in PBS at pH 7.2 .Fresh trypsin was prepared for each day test.

2.2.6.2.3 Hemagglutination Inhibition Procedure:

The method used is that described by WHO (2002). A volume of 25µL of PBS was dispensed into each well of a plastic U-bottomed microtitre plate. Then 25µL of the tested serum is added into the first well of each row of the plate.

A serial twofold dilution was made of 25µL volumes of the serum across the plate followed by the addition of 25µL of 4 HAU containing virus suspension to each well and left for a bout 30 minutes at room temperature to accomplish antigen-antibody interaction. Subsequently a 25µL of 1% (v/v) chicken RBCs was added to each well and after gentle mixing, the RBCs were allowed to settle for about 40 minutes at room temperature.

2.2.6.2.4 Interpretation of results:

The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen and the validity of results were assessed against a negative control serum. The HI titres was regarded as being positive if there is inhibition at a serum dilution of 1/16 or more against 4 HAU of antigen (OIE. 2005).

CHAPTER THREE
RESULTS

3.1. Agar gel-immunodiffusion test (AGID):

3.1.1. Preparation of Matrix and Nucleoprotein antigens for the AGID test:

Hemorrhage, edema and thickness were observed on the harvested chorioallantoic membranes (CAMs) which were inoculated with AI virus (H5N1).These antigens when tested with hyperimmune serum gave strong precipitation lines.

3.1.2 Serologic Survey for type A influenza antibodies using AGID test:

Two hundred and twenty two serum samples collected from humans were tested by AGID test to investigate and determine the presence of type A influenza antibodies.

As demonstrated in (Table 4) there were 76 (34.2%) samples out of the 222 samples positive to type A. And as Table 5 shows, the range of ages for the positive results extended below 20 and over 60 years old, which means that all ages, are exposed to infection with influenza A virus.

Table 4: Results of testing human sera by AGID test for the detection of influenza A antibodies:

	Frequency	Percent
Negative	146	65.8
Positive	76	34.2
Total	222	100.0

Table 5: Cross tabulation between Age and results of AGID test for determination of influenza A antibodies:

	AGID		Total
Age	Negative	Positive	
Less than 20	9	3	12
20 – 30	53	32	85
31 – 40	47	17	64
41 – 50	19	16	35
51 – 60	16	6	22
More than 60	2	2	4

Total	146	76	222

Table 6 shows the prevalence of type A influenza antibodies in human sera which was found to be 34.2% in the whole tested sera, and 46.9%, 62% in Khartoum and Omdurman respectively.

Table 6: Cross tabulation between Location and AGID test results for influenza A antibodies in human sera:

Location	AGID		Total
	Negative	Positive	
Khartoum	96	45(46.9%)	141
Omdurman	50	31(62%)	81
Total	146	76(34.2%)	222

3.2 Hemagglutination test for dilution of virus and preparation of virus 4 HAU:

The results of hemagglutination test were obtained to determine AI viruses' titers and the 4 HAU which should be used in HI test. As Table 7 shows the titer of H5N1 was found to be 7 log 2 and therefore the 4 HAU was calculated as 5 log 2. On the other hand, the titer of H7N3 was 6 log 2 and the 4 HAU was 4 log 2.

Table 7: Results of influenza A virus titration and dilution of the virus to prepare 4 HAU(s) for HI tests:

AI virus	Titer	4 HAU
H5N1	7 log 2	5 log 2
H7N3	6 log 2	4 log 2

3.3 Hemagglutination inhibition test:

To subtype the positive samples, that obtained by AGID test, hemagglutination inhibition (HI) test was applied using H5N1 and H7N3 avian influenza viruses as antigens.

Table 7 concluded the results of all sera, which were tested for AI (H5) antibodies, and it was found that there were 13 (17.1%) positive serum samples out of 76 samples. While Table 8 showed that 8 of these positive samples were from Khartoum and the rest 5 were from Omdurman. And when the 76 serum samples tested again against AI (H7) antibodies there were only 5(6.57%) positive samples, as shown in Table 9, three of them from Khartoum and the other two were from Omdurman, as in Table 10.

Table 8: Results of HI test for type A avian influenza AGID positive humans sera using avian influenza H5N1 as antigens.

	Frequency	Percent
Negative	63	82.9
Positive	13	17.1
Total	76	100.0

Table 9: Cross tabulation between Location and results of HI test for collected human sera using avian influenza H5N1 virus as antigen.

Location	HI for H5		
	Negative	Positive	Total
Khartoum	133	8	141
Omdurman	76	5	81
Total	209	13	222

Table 10: Results of HI test for avian influenza type A AGID positive humans sera using avian influenza H7N3 as antigens.

	Frequency	Percent
Negative	71	93.43
Positive	5	6.57
Total	76	100.0

However, Tables 11 and 12 summarize the cross tabs between age and the HI test results of H5 and H7 respectively, and from these two tables we can realize that there was no H5 infection under 20 years old persons, and the majority(11 out of 13)of infections were ranged between 20 and 50 years old. But in case of H7 there was one infection under 20 years and the range of infection (4 out of 5) was between20 and 40 years.

Table 11: Cross tabulation between Age and results of HI test for collected human sera using avian influenza H5N1 virus as antigen.

	HI for H5		
Age	Negative	Positive	Total
Less than 20	12	0	12
20 - 30	79	6	85
31 - 40	63	1	64
41 - 50	31	4	35
51 - 60	21	1	22
More than 60	3	1	4
Total	209	13	222

Table 12: Cross tabulation between Age and results of HI test for collected human sera using avian influenza H7N3 virus as antigen.

	HI for H7		
	Negative	Positive	Total
Age			
Less than 20	11	1	12
20 – 30	82	3	85
31 - 40	63	1	64
41 – 50	35	0	35
51 – 60	22	0	22
More than 60	4	0	4
Total	217	5	222

Table 13: Titers of antibodies detected by HI test against H5 and H7 avian influenza subtypes in the positive sera collected from humans.

AI virus	2 log 2	3 log 2	4 log 2	5 log 2	6 log 2	Total
H5N1	0	0	0	11	2	13
H7N3	0	1	0	4	0	5

Figure1: Results of AGID test for detection of influenza A antibodies in humans sera.

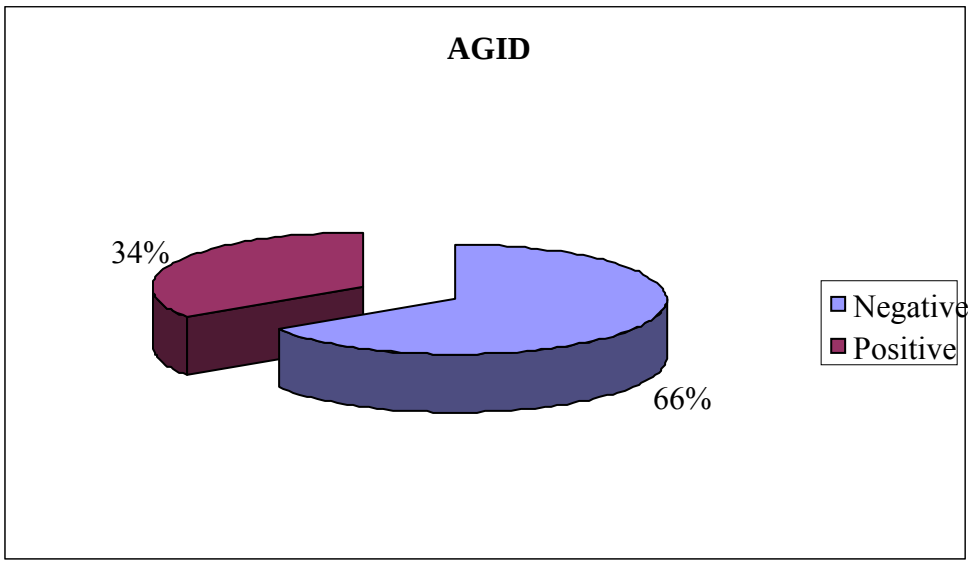


Figure 2: Comparison between avian influenza A antibodies positive and negative results tested by AGID and location of serum samples.

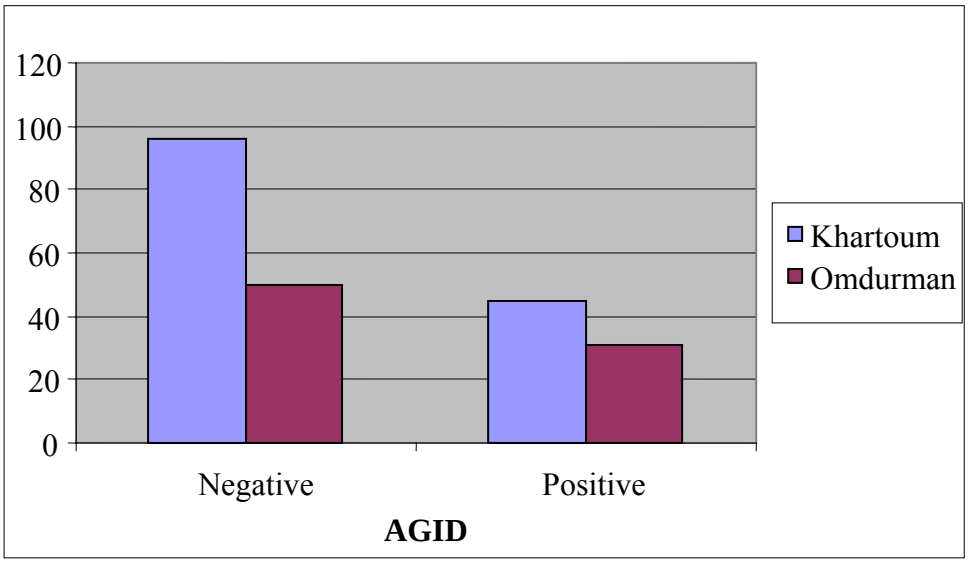


Figure 3: Cross tabulation between Age and avian influenza A antibodies detected by AGID test in human sera.

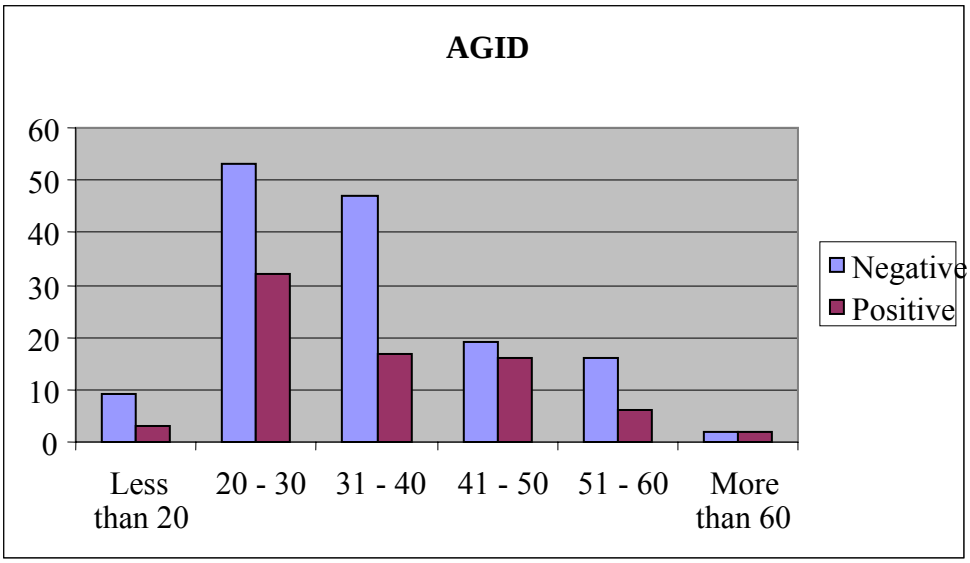


Figure 4: Cross tabulation between Age and results of HI test for collected human sera using avian influenza H5N1 virus as antigen.

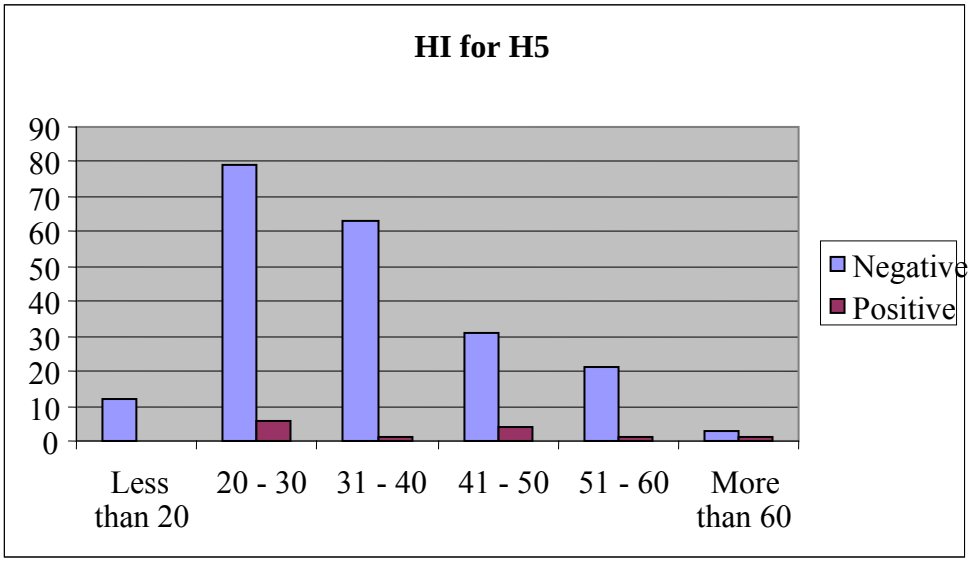


Figure 5: Cross tabulation between Age and results of HI test for collected human sera using avian influenza H7N3 virus as antigen.

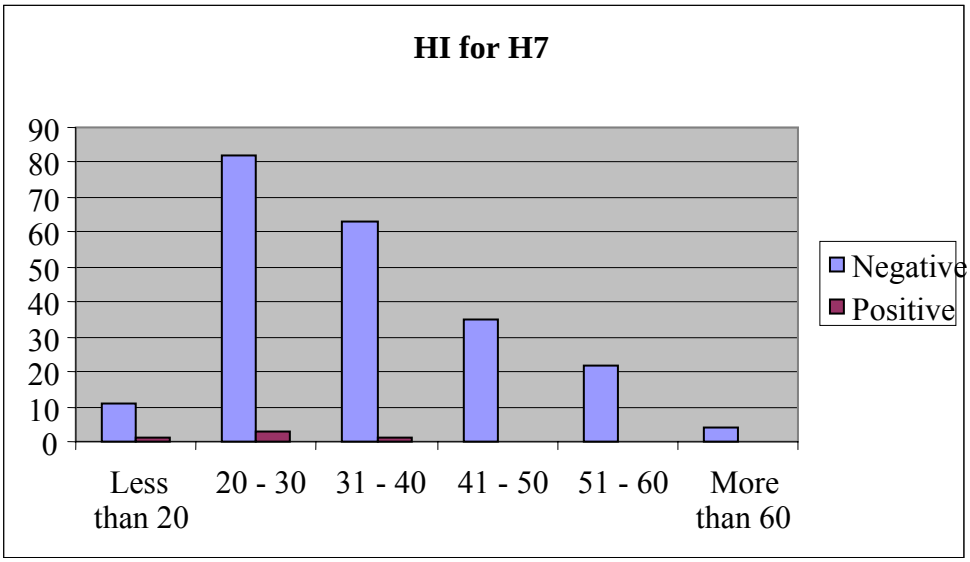


Figure 6: Cross tabulation between Location and results of HI test for collected human sera using avian influenza H5N1 virus as antigen.

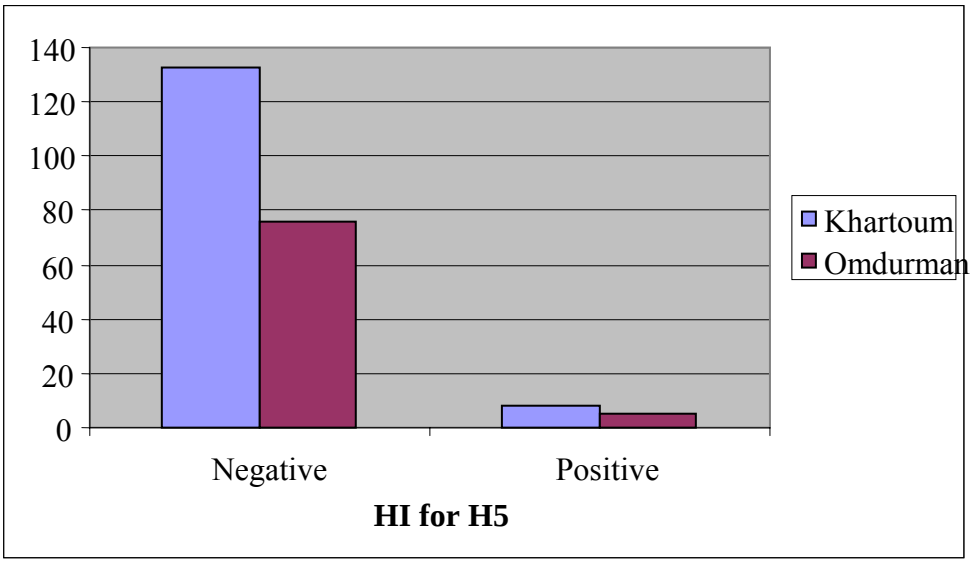
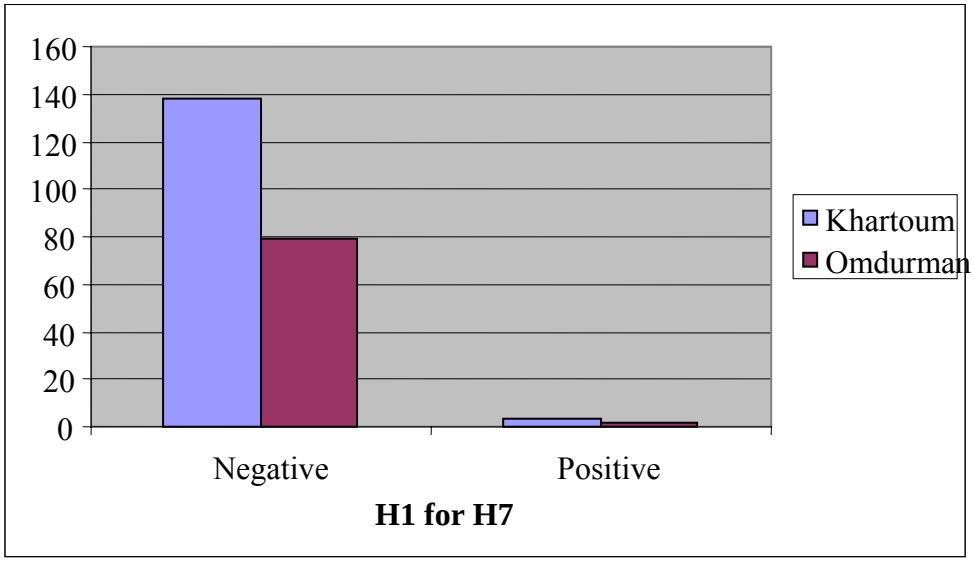


Figure 7: Cross tabulation between Location and results of HI test for collected human sera using avian influenza H7N3 virus as antigen



CHAPTER FOUR

DISCUSSION

Human influenza viruses are members of Orthomyxoviridae family, which consists of the genera: influenza A, B, and C virus, and Thogoto virus. In humans, only influenza A and B viruses are of epidemiological interest (Kamps, Hoffmann and Wolfgang, 2006).

Avian influenza (AI) is a highly contagious disease caused by type A influenza viruses that are members of the genus Influenza A virus. Infection with low-pathogenic avian influenza viruses (LPAIV) usually is asymptomatic or it induces mild respiratory symptoms. Infection with highly pathogenic avian influenza viruses (HPAIV) is characterized by the sudden onset of fatal systemic disease in susceptible species (OIE, 2005). Avian influenza A viruses, including those that are highly pathogenic in poultry, have not previously been associated with respiratory disease in humans, although H7 viruses have been associated with unrelated cases of human conjunctivitis (Webster, Geraci , Petursson and Skirnisson., 1981; Kurtz , Manvell and Banks, 1996).

In the last decade influenza diseases attracted a great world attention, since the first studied and documented human infection with H5N1 avian influenza virus that occurred in a 3-year-old male resident of Hong Kong who died in May 1997 of acute respiratory distress syndrome and complications.

According to the serological survey that was recently done (Wegdan et al., 2007), it can be concluded that there is a great widespread of AI among chicken in Sudan. Wegdan et al(2007) detect AI virus type A antibodies in 911 serum samples out of 1054 that were collected from

young, growing and adult unvaccinated chicken flocks at Khartoum State, Central, Northern, Eastern and western Sudan during 2003 to early 2006.

In the present study a total of 222 serum samples were collected from human, without apparent clinical manifestations of influenza or influenza-like diseases, by three different laboratories of Sudan Atomic Energy Commission (SAEC) in Khartoum (n=141) and Omdurman (n=81).

All collected sera were tested by AGID test to investigate the prevalence of type A influenza antibodies in Khartoum state. Over all results showed that: 76 serum samples out of 222(34.2%) were positive for influenza type A antibodies. Out of the positive sera (76), a number of 45(59.2%) samples were from Khartoum, while the rest 31(40.8%) samples were from Omdurman. The variation in the seroprevalence of AI in Khartoum and Omdurman may be attributed to the difference between the numbers of samples, since only 81 serum samples were from Omdurman.

The AGID assay is one of the gold standard tests that usually used to detect antibodies against type-specific M and NP antigens of type A influenza virus. Despite of the AGID test specificity, it has lower sensitivity, and commonly takes about 48–72 hours to obtain results (WHO, 2005).

Salim (1970), used complement fixation test (CFT), which is also used to detect antibodies against influenza type A viruses, to estimate the real incidence of influenza in the population of the three towns of Khartoum, Omdurman and Khartoum North. He found that out of 192 samples, there were 123 (64%) with a titer of 1\10 or more. CF titer is presumptive evidence of acute infection with influenza A virus. The variation in the two results may be due to the fact that Salim`s samples were collected

from acute ill patients with some or all of influenza symptoms and not randomly like ours.

The hemagglutination inhibition test was performed, in the present work, for sub-typing of AGID positive sera using H5N1 and H7N3 viruses as antigens .The antibodies to the two avian influenza A subtypes were detected in both Khartoum and Omdurman serum samples. The results revealed that, 13 out of the 76(17.1%) samples, which were positive by AGID, were positive for H5 when HI test was applied. And only 5(6.57%) samples were positive when tested by HI test using H7N3 as antigen.

From the 45 serum samples collected in Khartoum, which were positive by AGID test, there were only 8 tested positive for H5, and from the 31 samples collected from Ummdorman and were positive to type A influenza, there were only 5 positive samples to H5.

These results confirm the concept that, HI test is unable to detect the lower levels of antibody that may be present in sera from H5N1 virus-infected individuals (Rowe et al., 1999). Rowe and coauthers detected H5-specific antibody in only one serum sample, from 16 individuals confirmed or suspected to be infected with H5N1 virus, with high neutralizing-antibody titers and could not detect the relatively low serum antibody titers that developed in many individuals following primary infection with avian H5N1 virus.

The results that obtained by HI test for H7, are consider low compared with those obtained by Meijer et al (2006) who detected antibodies in high titers against A H7 virus in human sera (49% of 508 persons exposed to poultry and in 64% of 63 persons exposed to A (H7) infected persons) using horse erythrocytes instead of chicken RBCs and 2HAU of virus suspension. Horse erythrocyte agglutination requires recognition of NeuGc_ (2, 3) Gal receptor (Ito et al., 1997), and sera from A (H7)

infected persons could inhibit this agglutination efficiently. In our HI test, we employed whole virus rather than split virus antigen, and other researchers have suggested that the HI test is more sensitive when split virus antigen is used. Also the choice of the ideal method for treating sera to inactivate inhibitors, which depends on the nature of inhibitor present and the virus, may increase the sensitivity of the results (Subbarao, Kawaoka, Ryan-Poirier, Clements, and Murphy., 1992).

This study was performed for the first time to detect antibodies against influenza A viruses in human sera collected from Khartoum state.

One of the observations from this study is that, all age groups, in this survey, were exposed to infection with influenza A virus and this may be attributed to the ability of person to person transmission of human type A influenza viruses. In contrast the infection with AI viruses is limited to the most socially active groups, those ranged between 20 and 50 years old individuals only, due to the need of close contact with infected birds to be transmitted.

Conclusion and Recommendations:

Conclusion:

The outcome of this study proved the prevalence, of type A influenza in a percentage of 34.2% in human in Khartoum state, and revealed that there is a remarkable presence of H5 and H7 avian influenza antibodies in human sera in Khartoum state population. Also this study indicated that AGID test is reliable on to detect antibodies against influenza type A, even in case of no apparent clinical signs.

Recommendations:

1. Since influenza disease became one of the major human health concerns, it is recommended to improve the methods of diagnosis and sero-survey of influenza in humans.
2. More studies are needed to determine the circulating subtypes of influenza A among humans and avian in Sudan.
3. Local production of laboratories reagents including antigens and antibodies is needed to improve diagnosis.
4. Finally, another survey could be done using split antigens and horse RBCs.

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